博士（人間科学）学位論文

Functional role of downstream utrophin enhancer for utrophin expression in the skeletal muscle

骨格筋のユートロフィン発現におけるユートロフィンエンハンサーの機能的役割

2008 年 7 月

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Preface

The studies presented in this doctor thesis have been carried out under the direction of Professor Kazuhiko Imaizumi at the Laboratory of Physiological Sciences, Faculty of Human Sciences, Waseda University. The doctor thesis is concerned with the functional role of downstream utrophin enhancer for the utrophin expression in the skeletal muscle. I wish to express my grateful gratitude to Professor Kazuhiko Imaizumi. His kind guidance, precious comments, continuous encouragements and valuable discussions are deeply appreciated. I also wish to express my sincere thanks to Dr. Shin'ichi Takeda and Dr. Yuko Suzuki (Department of Molecular Therapy, National Institute of Neuroscience (NCNP), Kodaira, Tokyo) for their pertinent comments, many useful suggestions and valuable discussions.

I further thank Professor Kaoru Tachiyashiki (Department of Natural and Health Sciences, Joetsu University of Education, Joetsu, Niigata) for her warm-hearted encouragement, Dr. Michihiro Imamura for giving the utrophin antibody, and all members of the NCNP (Drs. Naoki Suzuki, So-ichiro Fukada, Akiyoshi Uezumi and Madoka Ikemoto, Kodaira, Tokyo) for technical assistance and useful suggestions. My thanks are also due to Dr. Ken Shirato (School of Medicine, Kyorin University, Mitaka, Tokyo) and Mr. Norio Motohashi (NCNP, Kodaira, Tokyo) for their continuous encouragement and support throughout the present study.

Finally, I thank for my family for their affectionate encouragement throughout this work.

Tokorozawa Jun Tanihata

May 27, 2008

Contents

1. Abstract

Duchenne muscular dystrophy is caused by the absence of the muscle cytoskeletal protein dystrophin. Utrophin is a 395 kDa cytoskeletal protein with a high degree identity with dystrophin at the amino acid levels and is an autosomal homologue of dystrophin, and overexpression of utrophin is expected to compensate for the dystrophin deficit. Recently, Takahashi et al. (2005) reported that the 5.4 kb 5'-flanking region of the utrophin gene containing the A-utrophin core promoter drives high levels of transgene expression in liver, testis, small intestine, submandibular gland, and colon, but not in heart and skeletal muscle. To clarify the regulatory mechanism of utrophin expression in the present study, we generated a nuclear localization signal-tagged LacZ transgenic (Tg) mouse, in which the LacZ gene was driven by the 129 bp downstream utrophin enhancer (DUE) and the 5.4 kb 5'-flanking region of the utrophin promoter. The levels of transgene mRNA expression in several tissues were examined by reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (q-RT-PCR), and cryosections of several tissues were stained with hematoxylin and eosin (H&E) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The expression patterns of the transgene were consistent with endogenous utrophin in several tissues including heart and skeletal muscle. Transgene expression was also up-regulated more in regenerating muscle than in non-regenerating muscle. Moreover, utrophin expression was augmented in the skeletal muscle of DUE Tg/X chromosome-linked muscular dystrophy (*mdx*) mice through cross-breeding experiments. We finally established cultures of primary myogenic cells from this Tg mouse and found that utrophin up-regulation during muscle differentiation depends on the DUE

motif. These cells provide a high through-put screening system for drugs that up-regulate utrophin expression in muscle cells. In conclusion, DUE is indispensable for utrophin expression in skeletal muscle and heart.

2. Introduction

It is generally accepted that skeletal muscles size depends upon a dynamic balance between catabolic (or atrophic) and anabolic (or hypertrophic) processes[1)]. Mass and fiber size in adult skeletal muscle is known to be regulated to changes in workload, activity or pathological conditions[2, 3)]. Muscle hypertrophy is associated with increased protein synthesis, while atrophy-induced by decreases in activity and load or by catabolic agents- enhances protein degradation pathway[1)]. However, it is unclear whether hypertrophy and atrophy pathways operate independently or whether they can regulate one another[1-3)].

Muscle atrophy occurs systematically in fasting and a variety of diseases ie., cancer, diabetes mellitus, AIDS, sepsis, cachexia, Cushing's syndrome and glucocorticoid treatment and in specific muscles upon denervation, immobilization, suspension or disuse[4-6)]. In these diverse conditions, the atrophying muscles show increased rates of protein degradation primarily through activation of ATP-dependent ubiquitin-proteasome pathway and a common series of transcriptional adaptations that together constitute an atrophy programs[3, 6)]. During atrophy, there is an increase in the amount of ubiquitin-protein conjugates and increased transcription of components of the ubiquitin pathway. The protein induced most dramatically during atrophy is the muscle-specific ubiquitin-ligase, atrogin-1 (MAFbx)[4, 7)]. mRNA for atrogin-1 increases 8-40 fold in at all types of atrophy studied, and this increase precedes the onset of muscle weight loss[4, 8)]. Moreover, knockout animals lacking atrogin-1 show a reduced rate of muscle atrophy after denervation[4, 9)].

On the other hand, induction of muscle hypertrophy by increased load is accompanied by

the increased expression of insulin-like growth factor (IGF-1)[1, 9, 10]. When IGF-1 levels were enhanced using a muscle-specific promoter in transgenic mice, increased muscle size resulted[11, 12]. Also, additional of IGF-1 *in vitro* to differentiated muscle cells promotes myotube hypertrophy, supporting the concept that hypertrophy can be mediated by pathways activated by autocrine or paracrine sources of IGF-1[11]. During adaptive hypertrophy in adult muscle and in IGF-1-induced myotube hypertrophy, serine/threonine kinase (Akt) is phosphorylated and activated. Additionally, hypertrophy elicits the direct and indirect targets downstream of Akt include glycogen synthesis kinase3 (GSK3), the mammalian target of rapamycin (mTOR), p70S6K, and eukaryotic initiation factor 4E binding protein 1 (4EBP-1), key regulatory protein involved in translation and protein synthesis[1, 4]. Moreover, IGF-1 treatment or over-expression of IGF-1 improved dystrophic phenotype in skeletal muscle by hypertrophic signal pathway via phosphorylation of Akt[1, 4, 13, 14].

Duchenne muscular dystrophy (DMD) is an X-linked progressive disorder caused by a defect in the DMD gene, which encodes dystrophin[15]. Dystrophin is a 427 kDa cytoskeletal protein that is normally located on the subsarcolemma and fixed by interaction with dystrophin-associated proteins (DAPs), some of which span the membrane[16-19]. This protein complex links the cytoskeleton of myofibers to the extracellular matrix to maintain the integrity of the sarcolemma. The lack of dystrophin in DMD causes a secondary loss of DAPs in the sarcolemma and leads to massive muscle necrosis, resulting in cardiomyopathy and early death. Unfortunately, there is no treatment available to stop the progression of this devastating neuromuscular disorder other than corticosteroids. Of the various therapeutic strategies for DMD being developed, up-regulation of utrophin has received considerable attention over the

last few years.

Utrophin is a 395 kDa cytoskeletal protein with a high degree identity with dystrophin at the amino acid level and is an autosomal homologue of dystrophin[20, 21]. It is ubiquitously expressed in most tissues. In embryonic and neonatal skeletal muscles, it is expressed both synaptically and extra-synaptically. In adult skeletal muscle, it is found mostly at the postsynaptic membrane of the neuromuscular junction (NMJ) and the myotendinous junction[22, 23].

X chromosome-linked muscular dystrophy (*Mdx*) mice completely lack the expression of dystrophin, but the signs and symptoms of DMD are not progressive until later in the course of the disease. This mild phenotype can be at least partly explained by up-regulation of utrophin at the sarcolemma[24, 25]. Additional studies have shown that utrophin is present in greater amounts in small caliber muscle fibers of *mdx* mice[26, 27] and small or regenerating muscle fibers of DMD patients[28, 29]. In contrast, utrophin null-*mdx* (dko) mice have a severe myopathic phenotype that is lethal within 20 weeks of birth[30].

Previous transgenic (Tg) experiments showed that overexpression of utrophin at the sarcolemma compensates for the lack of dystrophin and ameliorates dystrophic phenotypes in dystrophin-deficient *mdx* mice, where components of DAPs had been restored[31-33]. Similarly, adenovirally transduced utrophin ameliorates dystrophic changes in *mdx* mice[34]. Yamamoto *et al.*[35] showed that the immune response to adenovirally transferred β-galactosidase (β-gal) expression evoked up-regulation of endogenous utrophin, resulting in partial improvement of *mdx* phenotypes[35].

In addition, utrophin expression is up-regulated with Ca^{2+} inflow into cytoplasm through

the damaged muscle cell membrane[36]. Further, among several DAPs, only neuronal NOS (nNOS) quickly dislocated from sarcolemma to the cytoplasm and its nNOS induced NO production in the cytoplasm during tail suspension, a model of unloading-induced muscle atrophy[37] and it is also well known that L-arginine, the NO substrate induced up-regulation of endogenous utrophin expression[38].

These data suggested the not only systemic up-regulation of utrophin in DMD patients may lead to an effective treatment for this devastating disorder but also possibility that the muscle atrophy was repressed by up-regulation of utrophin expression. However, the regulatory mechanism of utrophin expression is not fully understood yet.

Transcriptional regulation of the utrophin gene is more complicated than previously pictured. Two full-length utrophin mRNAs, A and B, which encode different N-termini, are driven by two distinct promoters[39, 40]. Both A- and B-utrophin mRNAs are expressed in a tissue-specific manner, and an immunohistochemical study showed that A-utrophin is expressed in the NMJ, choroid plexus, pia mater, and renal glomerulus and tubule. On the other hand, B-utrophin is expressed in vascular endothelial cells[25]. Several short C-terminal utrophin isoforms have been also reported, as found in dystrophin[41]. To elucidate the transcriptional regulation of the utrophin gene, a more powerful tool is engineering an *in vivo* mouse model carrying a reporter gene.

Recently, Takahashi *et al.*[42] generated a transgenic mouse (Gnls) in which the LacZ gene was driven by the 5385-bp 5'-flanking region containing the A-utrophin promoter[42]. Expression of β-gal protein and mRNA derived from the transgene coincided well with the endogenous expression of utrophin in liver, testis, colon, submandibular gland, and small

intestine, but β-gal expression was extremely low in skeletal and cardiac muscle. These results suggested that muscle repressor elements may be present in the 5385-bp region or that another regulatory element outside the region might be required for the expression in striated muscle. Comparable results were found by Weir *et al.*[43] by using a transgene covering 3.8 kb of the mouse promoter region, which included the 1.3-kb reporter sequence characterized by Dennis *et al* [39].

The downstream utrophin enhancer (DUE) region was identified by Galvagni and Oliviero[44] and located about 9 kb downstream of the second intron. The upstream utrophin promoter is under the control of DUE[24, 44]. In the present study, therefore, we generated transgenic mice (DUE Tg) in which DUE was added upstream of the 5385-bp 5'-flanking region and analyzed the expression pattern of the transgene in several tissues. The present study found that the LacZ genes were expressed in skeletal and cardiac muscles. These data are very relevant to finding a way to up-regulate utrophin expression, and DUE mice as well as the primary cells derived from the mice are available for that purpose.

3. Material and Methods

3.1. Construction of transgene and generation of transgenic mice

To further investigate the utrophin A promoter activity in report of Takahashi *et al.*[42], genomic fragments containing the 5' end of the mouse A-utrophin gene were cloned from a 129Sv mouse genomic library (Stratagene, Texas, USA). One clone contained the 5385 bp 5'-flanking region of the A-utrophin gene, the complete exon 1A, and the first 59 bp of the exon 2 untranslated region. The genomic fragment was fused in-frame to an nls (from SV40T antigen)-tagged LacZ gene[45] (pCMVb, Clontech, California, USA), followed by a rabbit β-globin polyA signal in Bluescript II (Stratagene, Texas, USA)[42]. Furthermore, we inserted the 129-bp utrophin enhancer region that is found in utrophin gene intron 2[44] upstream of this fragment (Fig. 1).

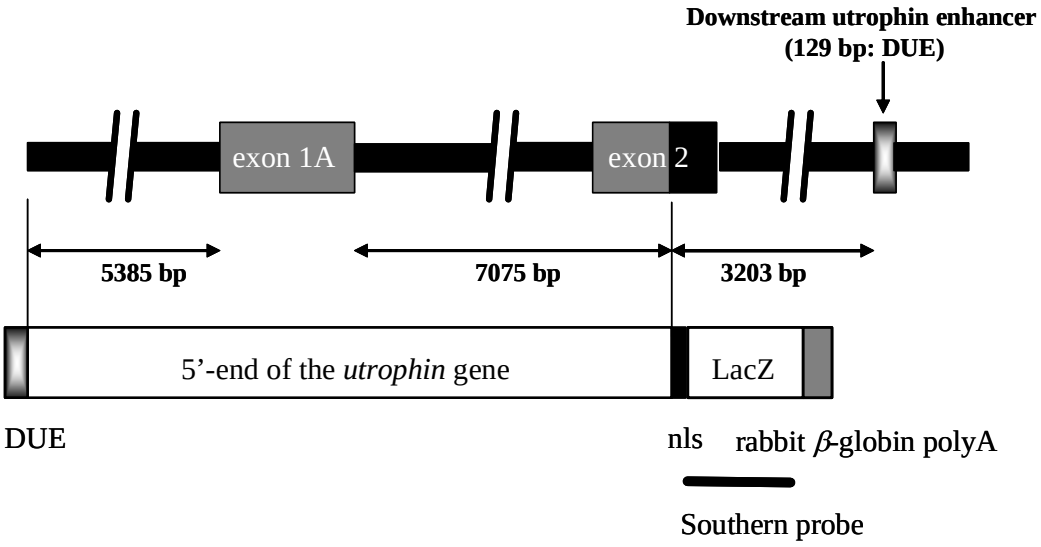

Fig. 1 Diagram of the transgene used in this study.

The genomic fragment (12.9 kb), which contained the 129-bp DUE, 5385-bp 5'-flanking region of the A-utrophin gene, exon 1A, intron 1, and the first 59 bp of exon 2 UTR, was fused in-frame to the nls-tagged LacZ gene. The black bar indicates the Southern probe used to determine genotypes.

The DNA fragment containing the transgene expression cassette was purified from agarose gel and injected into fertilized C57BL/6J eggs by YS Institute (Utsunomiya, Tochigi, Japan) (Table 1).

Table 1 Summary of generation of Tg mice.

	Number
implanted eggs	**523**
F_0	**129**
Tg positive F_0	**2 (♂ 1, ♀ 1) ⟶ line 1, line 2**

We obtained two transgenic F_0 (downstream utrophin enhancer/A promoter-nls LacZ transgenic mice), and two transgenic lines were established by mating the founders with C57BL/6J mice (B6) (Fig. 2).

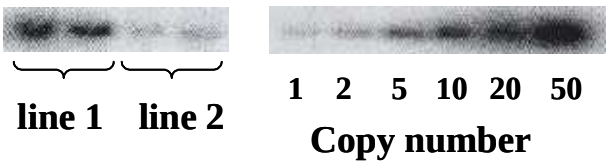

Fig. 2 Determination of copy numbers in Tg mice by Southern blotting.

The vector plasmids containing the transgene served as a standard to estimate the number of copies of the transgene.

To obtain transgenic *mdx* (Tg/*mdx*) male mice, we mated Tg male mice with *mdx* female mice (Fig. 3).

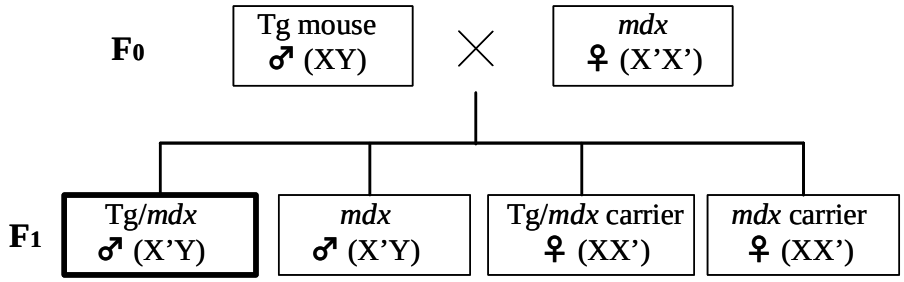

Fig. 3 Generation of Tg/*mdx* mice.

The bold box indicates the Tg/*mdx* male mice used in this study.

3.2. Animals

B6 and *mdx* mice aged 5-12 *weeks*, Tg mice and their wild littermates aged 3-18 *weeks*, and Tg/*mdx* mice aged 3-7 *weeks* were used. All animals were housed in a separate room at a temperature of 20-22℃ and under an artificial lighting regime (12 *hour* light/12 *hour* dark). Animals were sacrificed by cervical dislocation. All protocols were approved by the Institutional Animal Care and Use Committee of the National Institute of Neuroscience and were performed in compliance with the "Guide for the Care and Use of Animals" of the Division of Laboratory Animal Resources.

3.3. Genotyping

Tg mice were screened by Southern blotting of genomic DNA from their tails. Genomic DNA was isolated using a lysis buffer (50 *mM* Tris-HCl, pH 8.0, 0.1 *M* NaCl, 20 *mM* EDTA, 1% SDS) with proteinase K (0.15 *mg/ml*) and pronase E (1 *mg/ml*) digestion. Genomic DNA (10 μg) was digested by BamH Ⅰ, separated on a 0.8% agarose gel, and transferred to Hybond-N+ membranes (Amersham Biosciences, Buckinghamshire, UK). A 3072-bp DNA

fragment of the LacZ gene was labeled with ^{32}P-dCTP as a Southern probe, and hybridized with membranes at 65℃ overnight. The membranes were then washed extensively (2×SSPE, 0.1% SDS; 1×SSPE, 0.1% SDS; 0.1×SSPE, 0.1% SDS) at 65℃ and analyzed by BAS 2500 (Fuji Film, Tokyo, Japan).

3.4. Isolation of total RNA from mice and myogenic cells

3- to 8-*week*-old Tg mice and their wild-type littermates were sacrificed, and tissues were isolated and rapidly frozen in liquid nitrogen. Total RNA was isolated from frozen tissues and myogenic cells using TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's protocol.

3.5. Reverse transcription (RT)-PCR and quantitative real-time PCR (q-RT-PCR)

RT was performed with 1.0 μg of total RNA using a QuantiTect® Reverse Transcription Kit (Qiagen, Hamburg, Germany) according to the manufacturer's protocol. PCR was performed using LacZ sense (5'-CGACATTGGCGTAAGTGAAG-3') and antisense (5'-ATCGCCATTTGACCACTACC-3') primers for 30 cycles (denaturation at 95℃ for 1 *minute*, annealing at 60℃ for 30 *seconds*, and extension at 72℃ for 1 *minute*). As a control for the generation of PCR products due to residual contamination of genomic DNA, an equivalent amount of RNA that had not been treated with RT was also processed in parallel. The RT-PCR products of all samples were compared with the levels of a housekeeping gene, 18s rRNA, amplified with the following primer pair: sense (5'-TACCCTGGCGGTGGGATTAAC-3') and anti-sense (5'-CGAGAGAAGACCACGCCAAC-3') primers. The levels of various cDNAs

were determined by q-RT-PCR using SYBR Green from ABI PRISM 7700 (Applied Biosystems, California, USA). Each result shows the average of 3 or 4 samples. The LacZ and 18s rRNA primer sequences are described above. A-utrophin: sense (5'-ATGGCCAAGTATGGGGACC TTG-3') and anti-sense (5'-GTGGTGAAGTTGAGGACGTTGAC-3') primers; myogenin: sense (5'-CATGGTGCCCAGTGAATGCAACTC-3') and anti-sense (5'-TATCCTCCACC GTGATGCTGTCCA-3') primers; and MEF2C: sense (5'-TGGACAACAAAGCCCTCAGCAG GT-3') and anti-sense (5'-AATCCCTGCTTCGTTCCCTCTGC-3') primers were designed for q-RT-PCR. 18s rRNA mRNA was amplified as an internal control.

3.6. Histochemical analyses

After Tg and wild-type mice were sacrificed, the cerebrum, cerebellum, submandibular gland, lung, liver, kidney, small intestine, colon, testis, tibialis anterior (TA) and gastrocnemius (GC) muscles, diaphragm, and heart were isolated and frozen in liquid nitrogen-cooled isopentane. Cryosections (7 μm) from several tissues were stained with hematoxylin and eosin (H&E) and with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Wako Chemicals, Tokyo, Japan) according to the method of Ishii *et al.*[46].

3.7. Immunohistochemical analyses

Serial transverse cryosections (7 μm) from different tissues were placed on slides, then dried and fixed in acetone for 10 *minutes* at –20°C. We carried out immunohistochemical analysis with a rabbit polyclonal antibody against human utrophin (UT-2) that recognizes amino acid positions 1768-2078[47]. The primary antibodies were detected with Alexa 488-labeled goat

anti-rabbit IgG (Molecular Probes, California, USA). The nucleus was stained with TOTO-3 iodide (Molecular Probes, California, USA). The NMJ was stained with Alexa 594-labeled α-bungarotoxin (α-BTX) (Molecular Probes, California, USA). Signals were recorded photographically with a laser-scanning confocal imaging system (TCSSP™, Leica, St. Gallen, Switzerland).

3.8. Cardiotoxin injection

To cause muscle degeneration, we injected 100 *μl* of cardiotoxin (CTX) of *Naja naja atra* venom (10 *μM* in saline, Wako Chemicals, Tokyo, Japan) into the right TA and GC muscles of 5- to 7-*week*-old Tg mice using a 29-gauge needle. The concentration of CTX was determined according to the method of Couteaux *et al*.[48]. CTX-injected Tg mice were sacrificed 1-14 *days* after injection. The CTX-injected and contralateral non-injected TA and GC muscles were isolated and frozen in liquid nitrogen-cooled isopentane. Cryosections (7 *μm*) were stained with H&E and X-gal. At the same time, serial cryosections (7 *μm*) were stained with UT-2 together with Alexa 594-labeled α-BTX. Total RNA was isolated from these frozen tissues.

3.9. Cell preparation and culture

Mouse-derived mononucleated cells were prepared from DUE and Gnls Tg mice and C57BL/6J mice according to the method of Rando and Blau[49]. Primary myoblasts were cultured alone with growth medium (GM): F-10 containing 20% FBS, 1% penicillin-streptomycin (Invitrogen, California, USA), and 2.5 *ng*/*ml* bFGF (Invitrogen, California, USA) in collagen-coated dishes (Iwaki, Tokyo, Japan) or chamber slides (Nalge Nunc, New York,

USA) coated with collagen type Ⅰ (Upstate, Massachusetts, USA). For differentiation, the medium was changed to a differentiation medium (DM; 5% horse serum in DMEM) and cultured 5 *days*. GM and DM were replenished every 24 *hours*[50].

3.10. Statistics

Statistical differences were determined by Student's unpaired *t*-test. All data are expressed as means±SEM. Statistical significance was defined as $p < 0.05$.

4. Results

4.1. Generation of downstream utrophin enhancer/A promoter-nls LacZ transgenic (DUE Tg) mice

Two F_0 'founder' mice were identified by Southern blotting analysis using a LacZ cDNA probe, and two transgenic lines were established (Table 1). The approximate numbers of transgene copies were about 15 in line 1 or about 2 in line 2 (Fig. 2). The levels of transgene expression in several tissues were determined by RT-PCR (Fig. 4) and q-RT-PCR (Fig. 5).

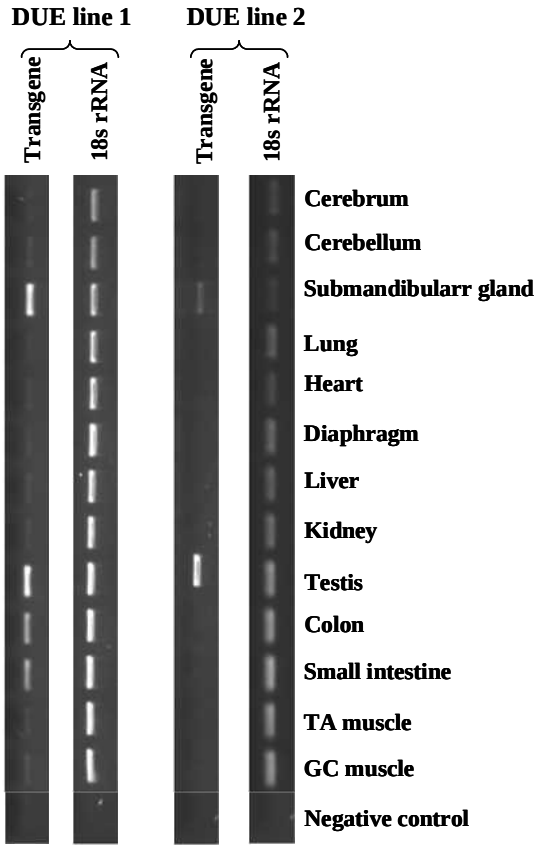

Fig. 4 Expression levels of transgene mRNA from several tissues in Tg mice. 18s rRNA are used as a control gene. Negative control consisted of RT mixtures in which reverse transcription enzyme was replaced with RNase-free water.

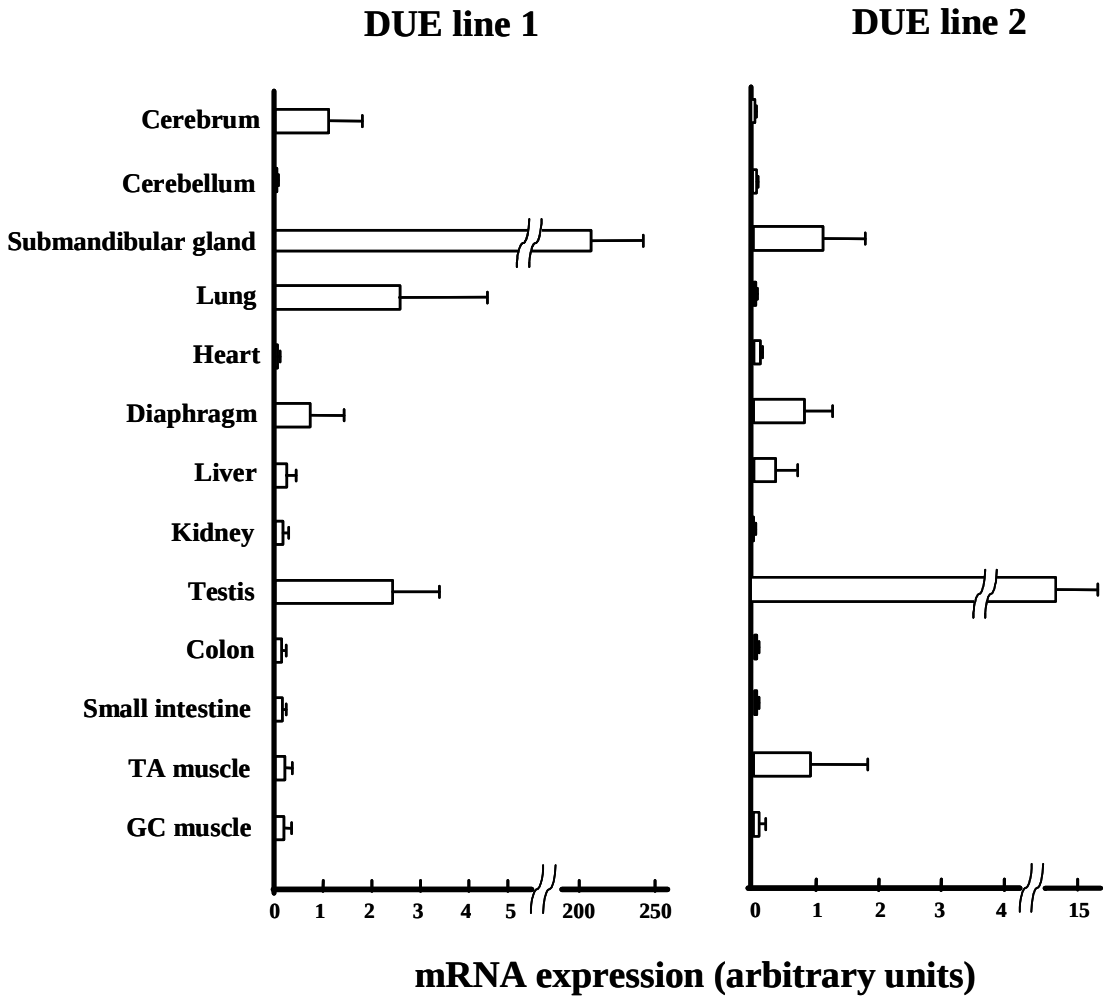

Fig. 5 Quantification of mRNA expression levels from several tissues in Tg mice.
18s rRNA are used as control gene. The ratio of the transgene to 18s rRNA is shown as the means±SEM of four independent experiments performed in triplicate.

High levels of transgene mRNA expression were detected in the submandibular gland, testis, lung, colon, and small intestine of DUE line 1 Tg mice and in the submandibular gland and testis of DUE line 2 Tg mice. The signals were weakly detected in other tissues of DUE line 2 Tg mice, probably because there were fewer copies of the transgene.

4.2. Comparison of β-gal and endogenous utrophin expression in DUE Tg mice

To compare β-gal expression derived from the transgene with endogenous utrophin expression, cryosections were stained with X-Gal, and then serial cryosections were stained with either H&E or UT-2, a polyclonal antibody against human utrophin[47] (Figs. 6-8). UT-2 recognized only the full-length A- and B-utrophin[47]. The present study showed that β-gal expression coincided well with endogenous utrophin expression in the liver, testis, colon, submandibular gland (Fig. 6), small intestine, kidney and lung (Fig.7).

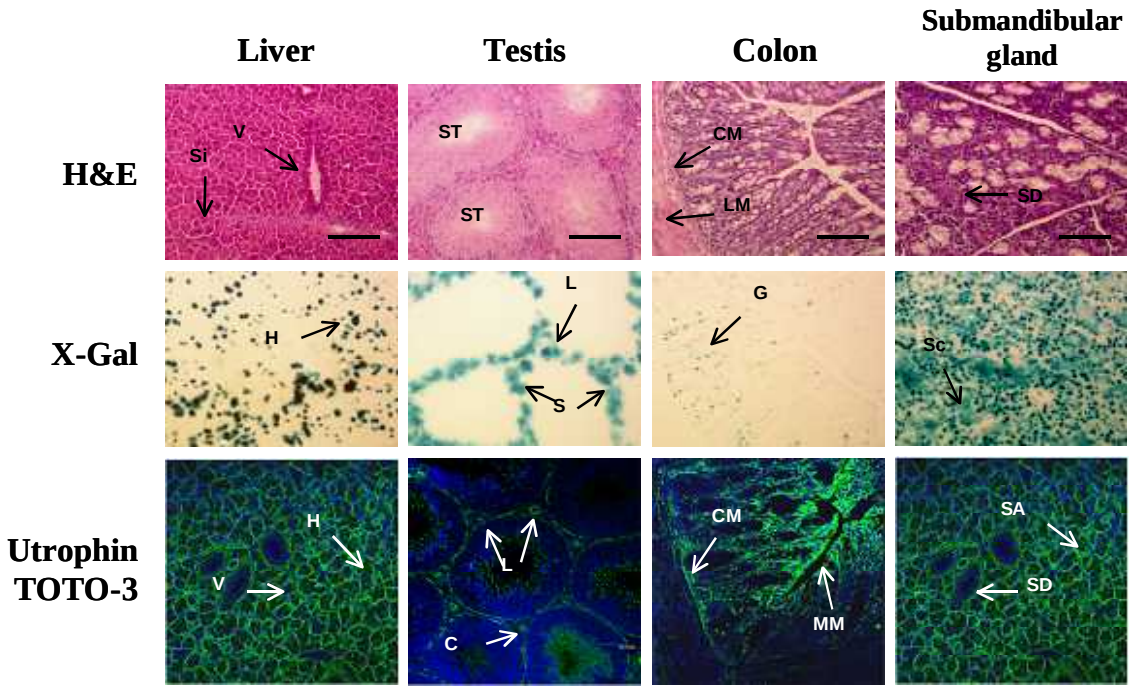

Fig. 6 Expression of endogenous utrophin and β-gal in DUE Tg mice.

Serial cryosections of tissues (liver, testis, colon and submandibular gland) from 7-*week*-old DUE line 1 Tg mice were stained with H&E (top), X-Gal (middle), and a polyclonal antibody against utophin (UT-2; green) (bottom). Nuclei were stained with TOTO-3 (blue). V, terminal hepatic venule ; Si, sinusoid ; H, hepatocyte ; ST, seminiferous tubule ; L, Leydig cell ; S, Sertoli cell ; C, capillary ; CM, inner circular muscle layer ; G, goblet cell ; LM, outer longitudinal muscle layer ; MM, muscularis mucosa ; SD, striated duct ; Sc, serous secretory cell ; SA, serous acinus ; Bar, 100 μm.

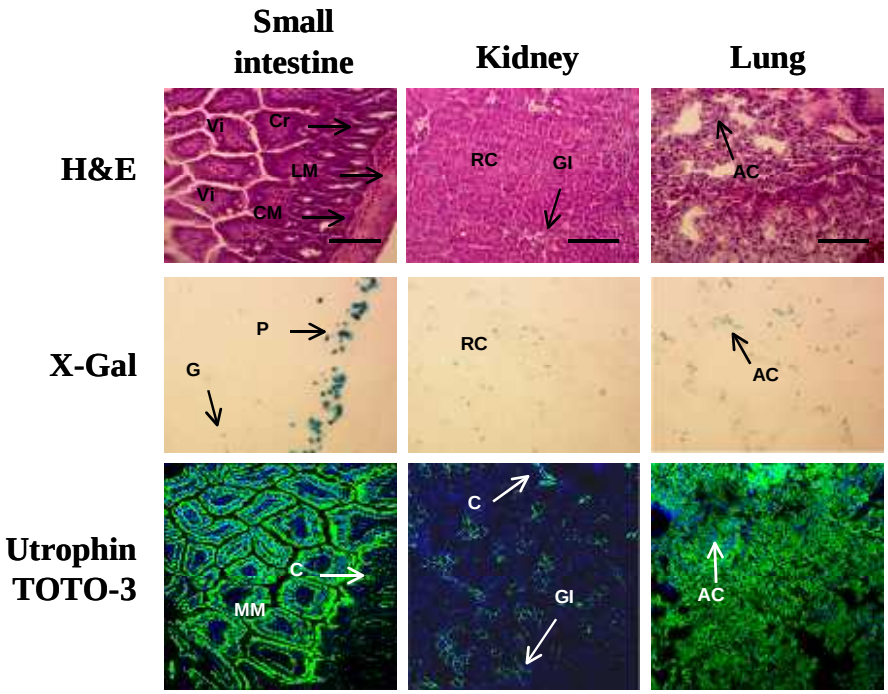

Fig. 7 Expression of endogenous utrophin and β-gal in DUE Tg mice.

Serial cryosections of tissues (small intestine, kidney, and lung) from 7-*week*-old DUE line 1 Tg mice were stained with H&E (top), X-Gal (middle), and a polyclonal antibody against utophin (UT-2; green) (bottom). Nuclei were stained with TOTO-3 (blue). Cr, crypt ; P, Paneth cell ; C, capillary ; RC, renal cortex ; Gl, glomerulus ; AC, alveolar cells ; Bar, 100 μm.

When compared with β-gal expression in Gnls Tg mice, expression levels of the transgene in DUE Tg mice were similar in the liver, testis, colon, submandibular gland, and small intestine, but were elevated in the kidney and lung. In addition, when transgene expression was examined at the protein level, the levels were higher in DUE line 1 Tg mice than those in line 2 Tg mice.

In the liver, the nuclei of hepatocytes, but not sinusoid lining cells, were strongly stained with X-gal, while endogenous utrophin was detected in the margins of hepatocytes along sinusoids and terminal hepatic venules.

In the testis, β-gal activity was found in Sertoli cells in the basal compartment of the seminiferous tubules, but not in the adluminal compartment, and in Leydig cells in the

interstitial supporting tissues between the seminiferous tubules. Consistent with this observation, endogenous utrophin signals were found along the basement membrane of the seminiferous tubules and Leydig cells.

In the colon, β-gal-positive nuclei were found in goblet cells in large intestinal glands. Endogenous utrophin signals were found along the basement membrane of large intestinal glands and the muscularis mucosa.

In the submandibular gland, the nuclei of both serous and mucous secretory cells were clearly stained with X-gal. The striated duct epithelia lacked the β-gal signal. Endogenous utrophin was detected along the basement membrane of serous and mucous acini, but not of striated ducts.

In the small intestine, β-gal-positive nuclei were found in goblet cells and Paneth cells which lie in epithelia of the bases of villi and crypts. Endogenous utrophin signals were found along the basement membrane of villi and crypts and in the muscularis mucosa.

In the kidney, β-gal-positive nuclei were found in the epithelia of cortical renal tubules, but not in glomeruli. It is not clear whether β-gal positive nuclei were present in proximal convoluted tubules, distal convoluted tubules, collecting tubules, or collecting ducts although endogenous utrophin was found along the basement membrane of cortical renal tubules, collecting ducts of the renal medulla and Bowman's capsules, and in glomerular capillaries.

In the lung, β-gal-positive nuclei were found in alveoli, but not in terminal bronchiole epithelia. It is not clear whether β-gal-positive nuclei were present in type I or type II pneumocytes. Endogenous utrophin was found in alveolar cells and terminal bronchiole epithelia. In these tissues, endogenous utrophin seemed to localize at the plasma membranes of

cells adjacent to the basement membranes.

4.3. β-gal expression in cerebrum, cerebellum, heart, and skeletal muscle of DUE Tg mice

In a previous study of Gnls Tg mice, we did not detect any β-gal expression in the cerebrum, cerebellum, heart, and skeletal muscle[42], but we found β-gal expression in these tissues in the DUE line 1 Tg mice (Fig. 8).

In the cerebrum, β-gal positive nuclei were found in ependymal cells of the choroid plexus and in fibroblastic cells of the pia mater along the basal membrane. Consistent with this observation, endogenous utrophin was detected in the choroid plexus and pia mater.

In the cerebellum, β-gal-positive nuclei were found in stellate and basket cells of the molecular layer, but not in fibroblastic cells of the pia mater, although endogenous utrophin is expressed in the pia mater of the cerebellum. Our results indicate that the distal utrophin enhancer cannot activate expression of the transgene in fibroblastic cells of the pia mater in the cerebellum, although it can enhance the expression in fibroblastic cells of the pia mater in the cerebrum.

In the heart, β-gal is expressed in myocardial nuclei located in the vicinity of intercalated disks. Endogenous utrophin expression was found in intercalated disks and T tubules of cardiac muscle.

In skeletal muscle, A-utrophin is expressed in NMJs, peripheral nerves, and larger blood vessels. We detected β-gal expression not only in myonuclei located close to NMJs but also in myonuclei remote from NMJs in DUE Tg skeletal muscles, but it is not clear whether or not β-gal-positive nuclei were present in nerves and blood vessels. Expression of the transgene was

not detected in the cerebrum, cerebellum, heart, and skeletal muscle in DUE line 2 Tg mice.

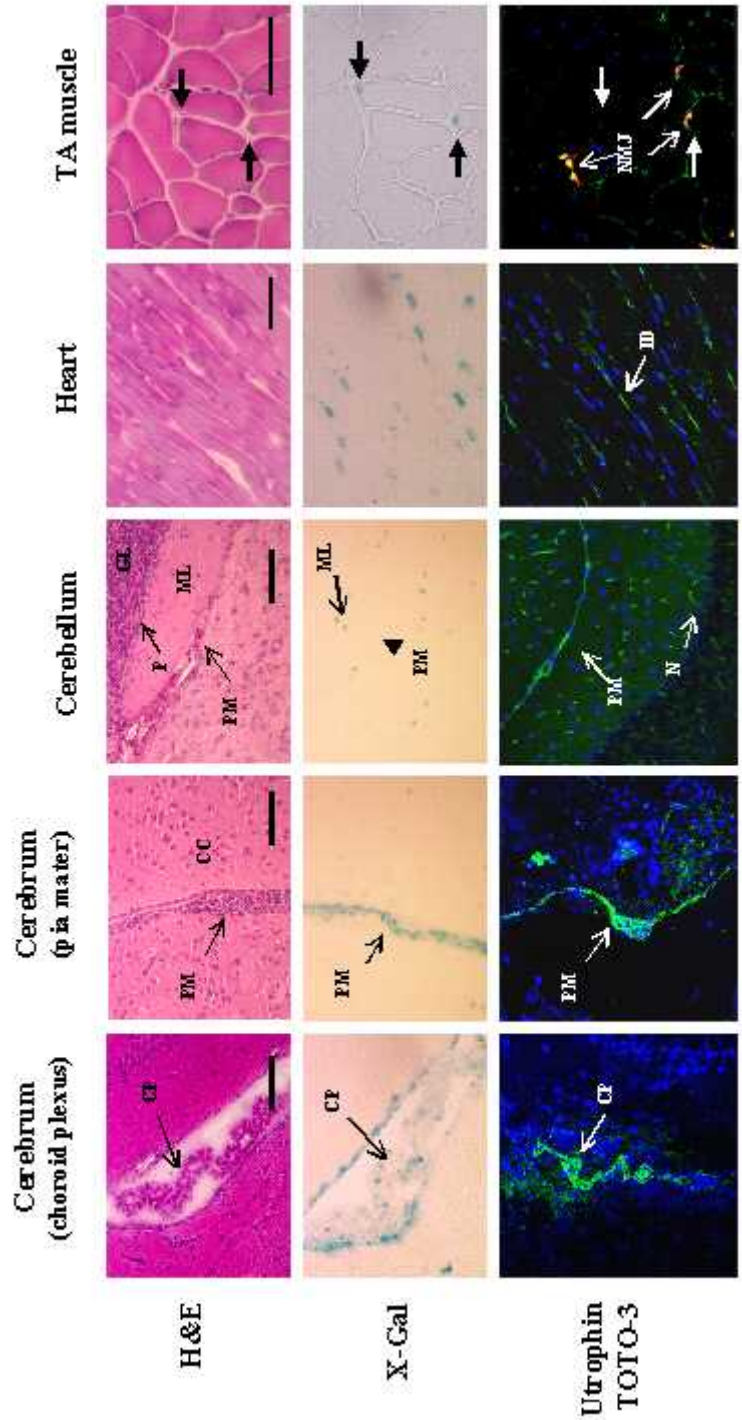

Fig. 8 Expression of endogenous utrophin and β-gal in DUE Tg mice.

Serial cryosections of tissues (choroids plexus and pia mater of cerebrum, cerebellum, heart, TA muscle) from 7-*week*-old DUE line 1 Tg mice were stained with H&E (top), X-Gal (middle), and a polyclonal antibody against utophin (UT-2; green) (bottom). Nuclei were stained with TOTO-3 (blue). The NMJs were identified with α-BTX (red) in TA muscle. CP, choroid plexus ; PM, pia mater ; CC, cerebral cortex ; N, neuron ; ML, molecular layer ; GL, granular layer ; P, Purkinje cell ; ID, intercalated disk ; NMJ, neuromuscular junction, Bar, 100 *μm*.

4.4. *β*-gal expression was augmented in CTX-injected and dystrophin-deficient DUE Tg mice

The present study examined expression of the transgene under a condition in which expression of endogenous utrophin is augmented. Recently, Galvagni *et al*. reported that the transcription of A-utrophin was activated in regenerating muscle under DUE control [24].

Therefore, the present study injected CTX into TA muscles of Tg mice to damage muscle fibers, and analyzed *β*-gal expression during muscle regeneration (Fig. 9). The *β*-gal signals were strongly detected in extrasynaptic regions at 5 or 7 *days* after CTX injection (Fig. 9A). Moreover, transgene mRNA levels were also elevated at 3 or 5 *days* after CTX injection (Fig. 9B). These transgene expressions coincided well with the expression of endogenous utrophin. It is interesting to note that transgene expression was also augmented in CTX-injected DUE line 2 Tg mice, although transgene expression was not detected in non-injected skeletal muscle of these mice (Fig. 10).

Utrophin was also up-regulated in the dystrophic process of *mdx* mouse skeletal muscle. To examine the transgene expression in dystrophin-deficient muscle, we mated DUE line 2 Tg male mice with *mdx* female mice (Fig. 3). Endogenous utrophin was overexpressed along the sarcolemma, and some myonuclei of TA and GC muscles of DUE line 2 Tg/*mdx* male mice were positive for *β*-gal (Fig. 11A). We also found slightly elevated mRNA levels derived from the transgene in TA and GC muscles of DUE line 2 Tg/*mdx* mice by RT-PCR (Fig. 11B).

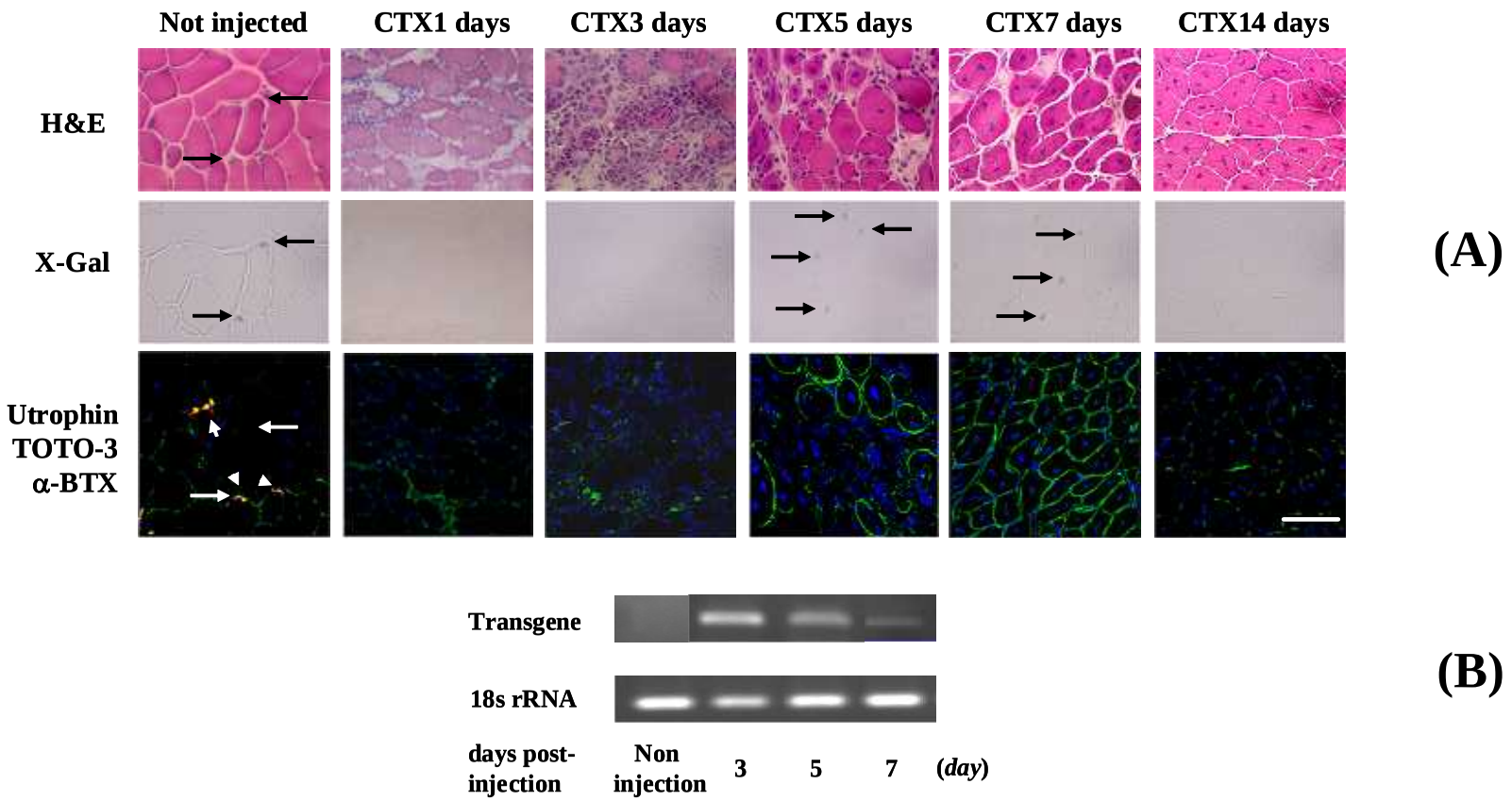

Fig. 9 Transgene expression during muscle regeneration in CTX-injected DUE line 1 Tg mice.

(A) Cryosections of muscles were stained with H&E (top), X-Gal (middle), and a polyclonal antibody against utophin (UT-2; green) (bottom). Nuclei were stained with TOTO-3 (blue). The NMJs were identified with α-BTX (red). TA muscle of DUE line 1 Tg mice at 0, 1, 3, 5, 7, or 14 *days* after CTX injection. Arrowhead, NMJ; bar, 100 *μm*. (B) Representative photomicrographs of EtBr-stained agarose gels depicting cDNA products for the transgene and 18s rRNAs from RT-PCR analysis of total RNA from CTX-injected TA muscle of DUE line 1 Tg mice at 0, 3, 5, or 7 *days* after CTX injection.

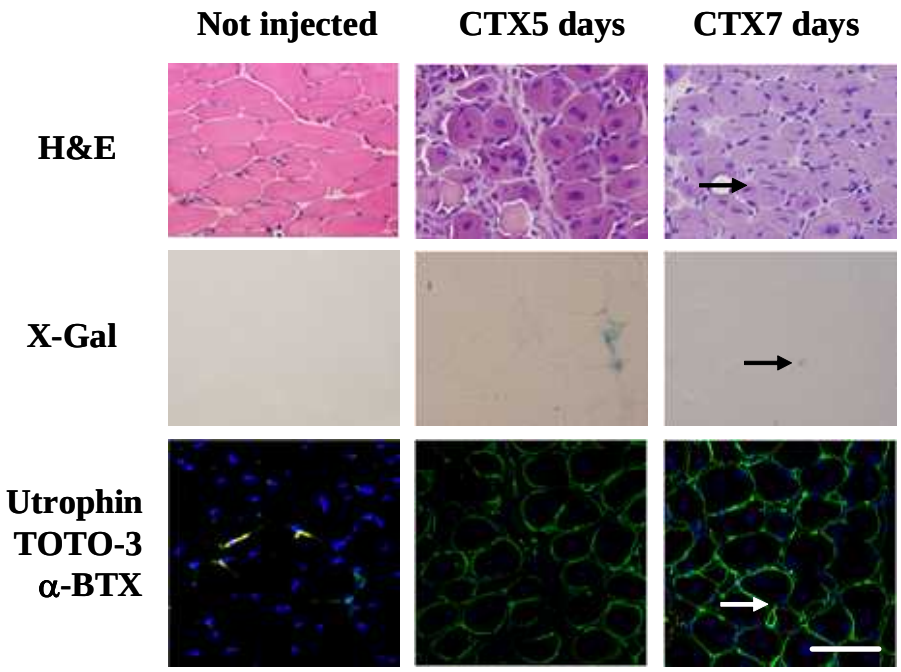

Fig. 10 Transgene expression during muscle regeneration in CTX-injected DUE line 2 Tg mice.

Cryosections of muscles were stained with H&E (top), X-Gal (middle), and a polyclonal antibody against utophin (UT-2; green) (bottom). Nuclei were stained with TOTO-3 (blue). The NMJs were identified with α-BTX (red). TA muscle of DUE line 2 Tg mice at 0, 5, or 7 *days* after CTX injection; bar, 100 μm

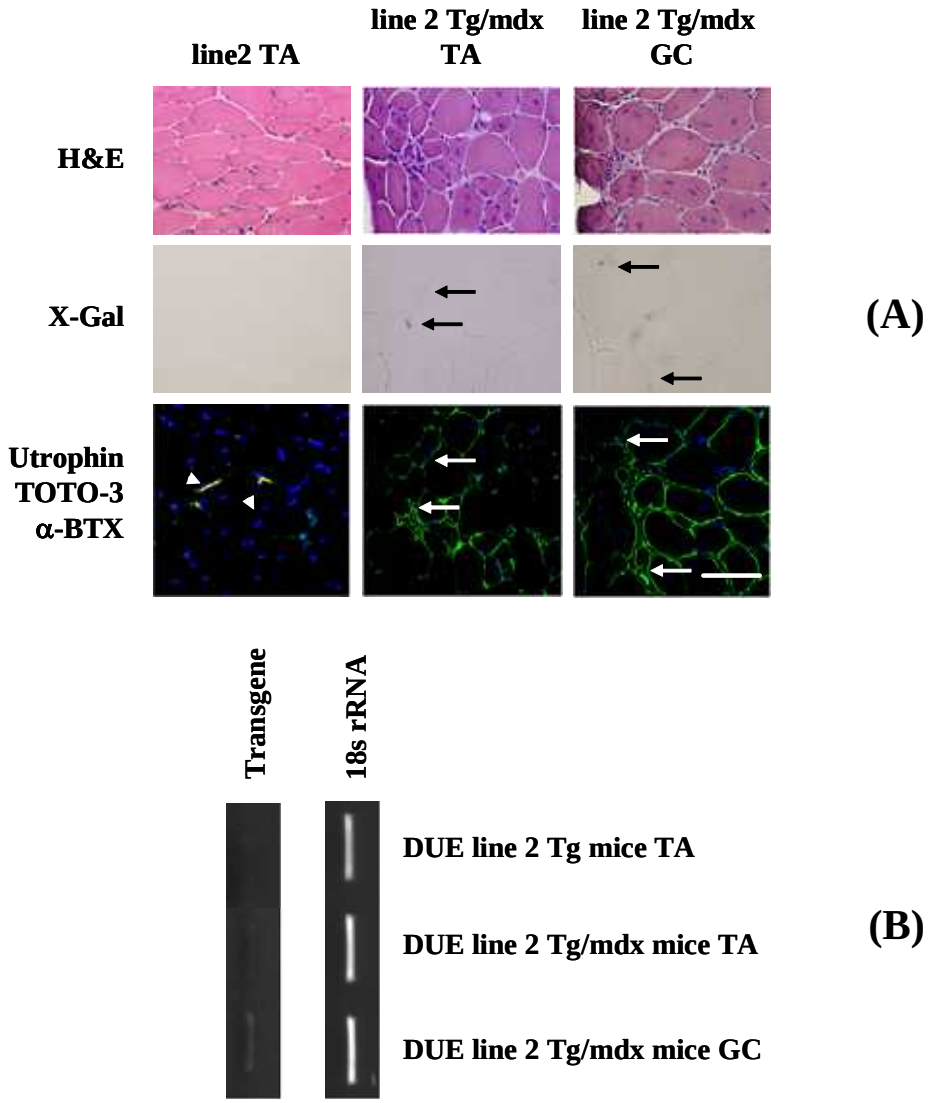

Fig. 11 Transgene expression in skeletal muscle of DUE Tg mice cross-bred with dystrophin deficient *mdx* mice.

(A) Cryosections of muscles were stained with H&E (top), X-Gal (middle), and a polyclonal antibody against utophin (UT-2; green) (bottom). Nuclei were stained with TOTO-3 (blue). The NMJs were identified with α-BTX (red). TA and GC muscles of DUE line 2 Tg/mdx mice. Arrow, β-gal-expressing nuclei; bar, 100 μm. (B) Representative photomicrographs of EtBr-stained agarose gels depicting cDNA products for the transgene and 18s rRNAs from RT-PCR analysis of total RNA from TA and GC muscles of DUE line 2 Tg/mdx mice. As a control, the TA muscle of DUE line 2 Tg mice was used.

4.5. Transgene expression was activated in late stage muscle differentiation *in vitro*

We cultured primary myoblasts from skeletal muscles of DUE Tg and Gnls Tg mice, and some of the cultures were induced to differentiate. Those cells were stained with X-Gal (Fig. 12). Expression of the transgene was detected in primary myoblasts of DUE Tg mice but not in those of Gnls Tg mice (Figs. 12, 13). In addition, the expression was up-regulated in myotubes in the DUE Tg mice, but not altered in myotubes of the Gnls Tg mice (Fig. 13). The up-regulation of the transgene was further investigated at the mRNA level, and it was found at a later stage of muscle differentiation. Very interestingly, the expression profiles of β-gal and endogenous utrophin were in accordance with that of MEF2C, but not compatible with that of myogenin.

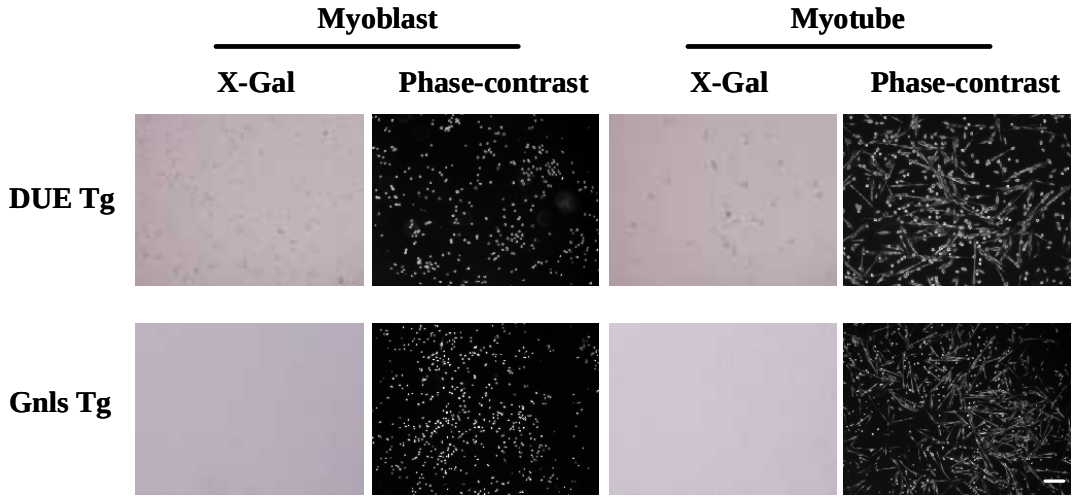

Fig. 12 Transgene expression in primary cultured myoblasts and myotubes from DUE Tg mice.

Primary cultured myoblasts and myotubes from DUE Tg mice and Gnls Tg mice were stained with X-Gal. Image of myotube after 3 *day*s in differentiation medium. Bar, 200 μm.

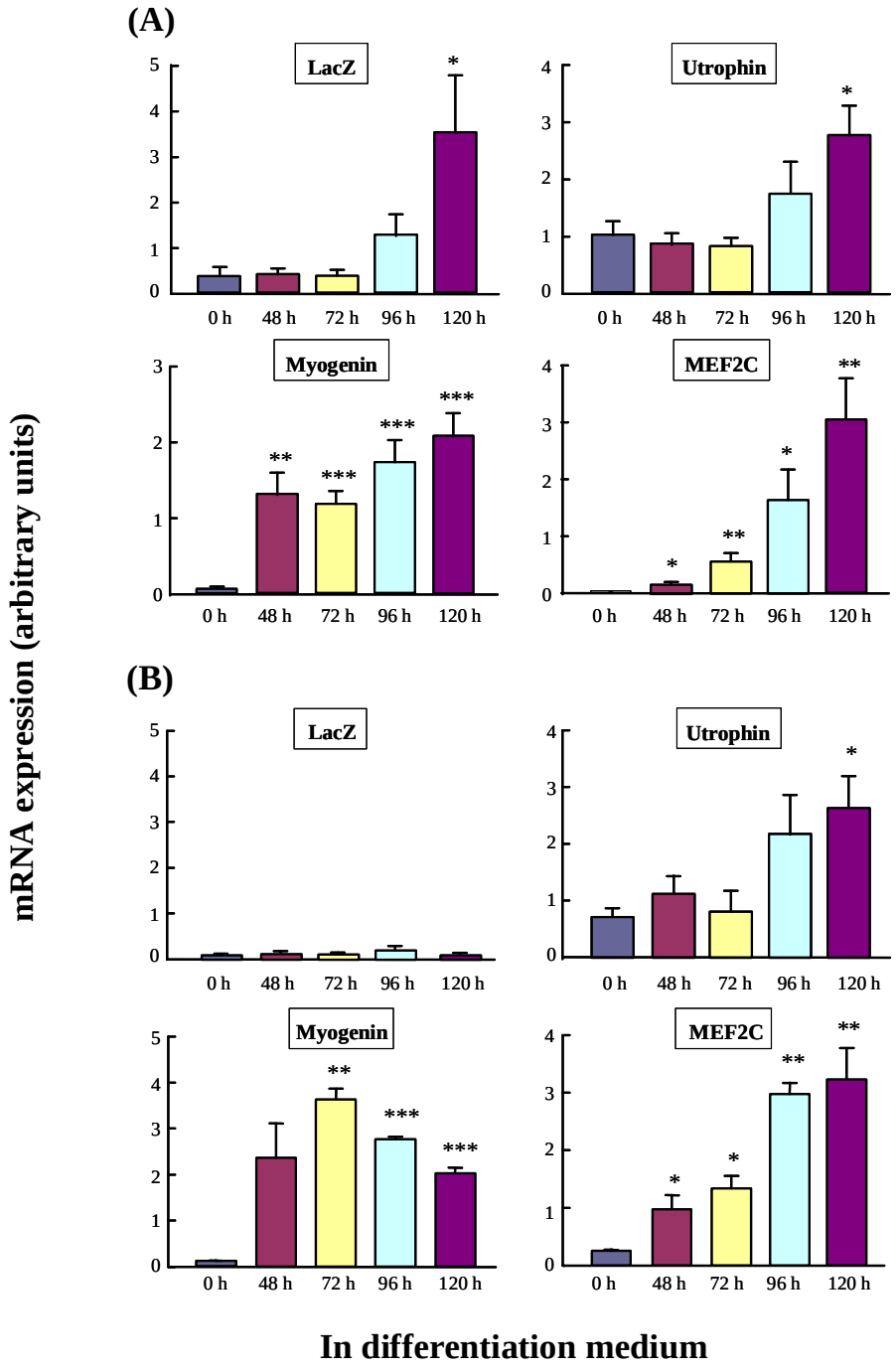

Fig. 13 Quantification of mRNA expression in primary cultured myoblasts and myotubes from DUE Tg mice and Gnls Tg mice.

(A) and (B) Quantification of q-RT-PCR products for transgene, A-utrophin, myogenin, and MEF2C optimized to expression of 18s rRNA in primary myoblasts and myotubes from DUE Tg mice and Gnls Tg mice. The ratios of the transgene, A-utrophin, myogenin, and MEF2C to 18s rRNA are shown as the means±SEM of four independent experiments performed in triplicate. *$P < 0.05$, ** $p < 0.01$ and ***$P < 0.001$ (*vs.* 0 time).

5. Discussion

Recently, Takahashi *et al.*[42] reported that a 5385-bp 5'-flanking region of the utrophin gene containing the A-utrophin core promoter drives high levels of transgene expression in liver, testis, colon, submandibular gland, and small intestine, but not in heart and skeletal muscle. The present study demonstrated that addition of DUE to the 5385-bp 5'-flanking region enabled transgene expression in a pattern that was almost identical to that endogenous utrophin expression (Tables 2, 3). Moreover, β-gal-expressing nuclei were basically located in the vicinity of the endogenous utrophin expression in heart and skeletal muscle.

In regenerating muscle of DUE Tg mice and skeletal muscle of DUE Tg/*mdx* mice, which lack dystrophin, the transgene expression was considerably up-regulated. Another research group also reported that utrophin transcription was controlled by DUE activity in regenerating muscle and that its activity was dependent on an AP-1 binding site[24]. They injected marcain into TA muscles of CD1 mice and found elevation of members of the AP-1 factor, *c-fos, fosB, fra-1, fra-2, c-jun, junB* and *junD*, but we also found distinct elevation of *c-fos* and *fra-1* mRNA in our regeneration system (unpublished observations).

We cultured primary myogenic cells from DUE Tg mice and found that transgene expression was up-regulated during the differentiation process. Moreover, these transgene expression patterns corresponded to the endogenous utrophin expression profile. This result indicates that the participation of DUE in utrophin expression during muscle regeneration might depend largely on DUE activity in the later stage of muscle differentiation. It is intriguing to note that transgene and endogenous utrophin expression patterns coincided with the expression

Table 2. Cells that express β-gal in Gnls Tg and DUE Tg mice and comparison with endogenous utrophin expression.

Tissue	Endogenous utrophin	β-Gal expression		
		Gnls	DUE line 1	DUE line2
Liver	Surface of hepatocyte	hepatocyte	hepatocyte	hepatocyte
Testis	BM of seminiferous tubule	Sertoli cell	Sertoli cell	Sertoli cell
	Leydig cell	Leydig cell	Leydig cell	Leydig cell
Colon	BM of large intestinal gland	Goblet cell	Goblet cell	Goblet cell
	muscularis mucosa	n.d..	n.d.	n.d.
Submandibular gland	BM of serous & mucous acinus	serous & mucous secretory cell	serous & mucous secretory cell	serous & mucous secretory cell
Small intestine	BM of villus & crypt	Paneth cell, goblet cell	Paneth cell, goblet cell	Paneth cell, goblet cell
	muscularis mucosa	n.d.		
Kidney	BM of cortical renal tubule	epithelial cell of cortical renal tubule	epithelial cell of cortical renal tubule	epithelial cell of cortical renal tubule
	BM of collecting duct in renal medulla			
	glomerulus	n.d.	n.d.	n.d.
Lung	alveolus	alveolar cell	alveolar cell	alveolar cell
	terminal bronchiole epithelium	n.d.	n.d.	n.d.
Cerebrum	choroid plexus	n.d.	epithelial cell of choroid plexus	n.d.
	pia mater		fibroblastic cell of pia mater	
Cerebellum	pia mater	n.d.	stellate cell and basket cell of molecular layer	n.d.
Heart	intercalated disk	n.d.	peripheral cell of intercalated disk	n.d.
	T tubule			
Skeletal muscle	nuromuscular junction	n.d.	peripheral cell of nuromuscular junction	n.d.
	myotendinous junction			
	regenerating muscle fiber			

The localization of endogenous utrophin is based on this study and previous studies[25, 38]. BM, basement membrane; n.d., not detected.

Table 3. Summary of β-gal expression in Gnls Tg mice and DUE Tg mice.

Visceral organs	β-gal expression		
	Gnls	DUE line 1	DUE line 2
Liver	++	++	±
Testis	+++	+++	+
Colon	++	++	+
Submandibular gland	+++	+++	+
Small intestine	+	+	±
kidney	±	+	+
Lung	+	++	±
Cerebrum	−	++	−
Cerebellum	−	++	−
Heart	−	++	−
TA muscle	−	+	−

Tg mice were sacrificed at 4-7 weeks old, and β-gal expression was examined in several tissues. No β-gal positive nuclei were found in non-transgenic littermates. β-gal expression levels are shown as: −, none; ±, trace; +, weak; ++, moderate; and +++, strong.

profile of MEF2C, but not that of myogenin. It has been already reported that MEF2C mediates the promoter activity of c-jun[51]. The MEF2C-c-jun pathway is one of the candidates to regulate utrophin expression via DUE. Analysis of the transcriptional factors that interact with DUE sequences, particularly the AP-1 site, would be very intriguing and should be clarified by a future study.

The present study also demonstrated that the addition of DUE augmented transgene expression not only in the heart and skeletal muscle but also in other tissues such as the cerebral pia mater and choroid plexus and the cerebellar choroid plexus and molecular layer. In addition, transgene expression was elevated in the kidney and lung of DUE Tg mice compared with that of Gnls Tg mice, although it is necessary to consider the difference in transgene copy numbers.

These results suggest that DUE activity is not muscle specific, and this suggestion is consistent with the data of Galvagni *et al.*[44]. In their report, a construct of DUE added to the utrophin promoter was transiently transfected to various cells and revealed that DUE enhanced utrophin promoter activity not only in C2C12 myoblasts but also in HeLa cells and RD cells.

However, the addition of DUE cannot fully explain the transcriptional regulation of utrophin. In the cerebrum and cerebellum, endogenous utrophin was expressed in the pia mater and choroid plexus. We found β-gal-positive nuclei in the cerebral pia mater along the basal lamina, but did not find many β-gal-positive nuclei in the cerebellum. There are several possibilities to explain this discrepancy. The first possibility is that the domain that regulates utrophin expression in the pia mater of the cerebellum is different from that for the pia mater in the cerebrum. The second possibility is that transcription of utrophin might be less active in fibroblastic cells of the pia mater of cerebellum than those of the cerebrum. However, a fundamental difference between fibroblastic cells in the cerebrum and those in the cerebellum has not been reported; further experiments are required to explain this discrepancy.

The present study demonstrated that DUE is necessary for utrophin expression in skeletal muscle, but the increase in the utrophin expression level was much larger than the transgene expression in regenerated muscle. Another research group also detected the increase in the abundance of A-utrophin protein in muscle from *mdx* mice but could not find any parallel elevation in the levels of utrophin transcripts[25]. Therefore, A-utrophin expression may also be regulated at the post-transcriptional level. Indeed, recent studies have shown that distinct cis-acting elements within the utrophin 3'-untranslated region (UTR) were important not only for controlling the stability of utrophin transcripts in muscle cells but also for targeting them to

specific subcellular locations[52, 53].

Post-translational levels are also important for utrophin expression through stabilization of the protein. DAPs such as dystrophin, β-dystroglycan, α-dystroglycan, and α-sarcoglycan have been linked to regulation by protein degradation mechanisms including the ubiquitin-proteasome pathway[54] and calpain-mediated proteolysis[55]. Inhibition of the proteasomal degradation pathway was found to rescue the expression levels of several DAPs in *mdx* mice[54]. Treatment of normal and DMD human myotubes with glucocorticoid induced utrophin protein without elevations in transcripts, and this was speculated to involve calpain inhibition[56].

It is likely that extrasynaptic expression of utrophin in skeletal muscle of DMD patients would ameliorate the dystrophic pathology, at least to some extent[32, 33]. The results of this report demonstrate that DUE is indispensable to utrophin expression in skeletal muscle and heart. To further investigate the up-regulation mechanisms of utrophin in both tissues, we need to search for transcription factors bound to DUE. In addition, we established primary myogenic cell cultures from DUE Tg mice and found that utrophin up-regulation depends on the DUE motif during muscle differentiation. These cells provide a high through-put screening system for drugs that can up-regulate utrophin expression in myogenic cells.

6. References

1. Trevor NS, Doreen D, Brian AC, Frank P, Yekatarina T, William OK, Michael G, George DY and David JG. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligase by inhibiting Foxo transcription factors. *Molecular Cell*, 14: 395-403 (2004)
2. Glass DJ. Signaling pathways that mediate skeletal muscle hypertrophy and atrophy. *Nat Cell Biol*, 5: 87-90 (2003)
3. Jagoe RT and Goldberg AL. What do we really know about the ubiquitin-proteasome pathway in muscle atrophy? *Curr Opin Clin Nutr Metab Care*, 4: 183-190 (2001)
4. Marco S Claudia S, Alex G, Carsten S, Elisa C, Anne P, Kenneth W, Stefano S, Stewart HL, and Alfred LG. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell*, 117: 399-412 (2004)
5. Lecker SH, Jagoe RT, Gilbert A, Gomes M, Baracos V, Bailey J, Price SR, Mitch WE and Goldberg AL. Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *FASEB J*, 18: 39-51 (2004)
6. Lecker SH, Solomon V, Mitch WE and Goldberg AL. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and diseases states. J Nutr, 129: 227S-237S (1999)
7. Gomes MD, Lecker SH, Jagoe RT, Navon A and Goldberg AL. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc Natl Acad Sci USA*, 98: 14440-14445 (2001)

8. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ and Yancopoulos GD. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol, 3: 1014-1019 (2001)
9. Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD and Glass DJ. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science, 294: 1704-1708 (2001)
10. DeVol DL, Rotwein P, Sadow JL, Novakofski J and Bechtel PJ. Activation of insulin-like growth factor gene expression during work-induced skeletal muscle growth. Am J Physiol, 259: E89-E95 (1990)
11. Coleman ME, DeMayo F, Yin KC, Lee HM, Geske R, Montgomery C and Schwartz RJ. Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice. J Biol Chem, 270: 12109-12116 (1995)
12. Musaro A, McCullagh K, Paul A, Houghton L, Dobrowolny G, Molinaro M, Barton ER, Sweeney HL and Rosenthal N. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet, 27: 195-200 (2001)
13. Barton ER, Morris L, Musaro A, Rosenthal N and Sweeney HL. Muscle-specific expression of insulin-like growth factor I counters muscle decline in *mdx* mice. J Cell Biol, 157: 137-147 (2002)
14. Grounds MD, Gadley HG, Gebski BL, Bogoyevitch MA and Shavlakadze T. Proceeding of

cross-talk between tumor necrosis factor and insulin-like growth factor-1 signaling in skeletal muscle. Clin Exp Pharmacol Physiol, (Epub ahead of print) (2008)

15. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C and Kunkel LM. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell,* 50: 509-517 (1987)
16. Ahn AH and Kunkel LM. The structural and functional diversity of dystrophin. *Nat Genet,* 3: 283-291 (1993)
17. Tinsley JM, Blake DJ, Zuellig RA and Davies KE. Increasing complexity of the dystrophin-associated protein complex. *Proc Natl Acad Sci USA,* 91: 8307-8313 (1994)
18. Campbell KP. Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage. *Cell,* 80: 675-679 (1995)
19. Ozawa E, Yoshida M, Suzuki A, Mizuno Y, Hagiwara Y and Noguchi S. Dystrophin-associated proteins in muscular dystrophy. *Hum Mol Genet,* 4 Spec No: 1711-1716 (1995)
20. Pearce M, Blake DJ, Tinsley JM, Byth BC, Campbell L, Monaco AP and Davies KE. The utrophin and dystrophin genes share similarities in genomic structure. *Hum Mol Genet,* 2: 1765-1772 (1993)
21. Grady RM, Teng H, Nichol MC, Cunningham JC, Wilkinson RS and Sanes JR. Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. *Cell,* 90: 729-738 (1997)
22. Khurana TS, Watkins SC, Chafey P, Chelly J, Tome FM, Fardeau M, Kaplan JC and Kunkel LM. Immunolocalization and developmental expression of dystrophin related protein in skeletal muscle. *Neuromuscul Disord,* 1: 185-194 (1991)

23. Ohlendieck K, Ervasti JM, Matsumura K, Kahl SD, Leveille CJ and Campbell KP. Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. *Neuron,* 7: 499-508 (1991)

24. Galvagni F, Cantini M and Oliviero S. The utrophin gene is transcriptionally up-regulated in regenerating muscle. *J Biol Chem,* 277: 19106-19113 (2002)

25. Weir AP, Burton EA, Harrod G and Davies KE. A- and B-utrophin have different expression patterns and are differentially up-regulated in mdx muscle. *J Biol Chem,* 277: 45285-45290 (2002)

26. Takemitsu M, Ishiura S, Koga R, Kamakura K, Arahata K, Nonaka I and Sugita H. Dystrophin-related protein in the fetal and denervated skeletal muscles of normal and mdx mice. *Biochem Biophys Res Commun,* 180: 1179-1186 (1991)

27. Matsumura K, Ervasti JM, Ohlendieck K, Kahl SD and Campbell KP. Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. *Nature,* 360: 588-591 (1992)

28. Helliwell TR, Man NT, Morris GE and Davies KE. The dystrophin-related protein, utrophin, is expressed on the sarcolemma of regenerating human skeletal muscle fibres in dystrophies and inflammatory myopathies. *Neuromuscul Disord,* 2: 177-184 (1992)

29. Nguyen TM, Ellis JM, Love DR, Davies KE, Gatter KC, Dickson G and Morris GE. Localization of the DMDL gene-encoded dystrophin-related protein using a panel of nineteen monoclonal antibodies: presence at neuromuscular junctions, in the sarcolemma of dystrophic skeletal muscle, in vascular and other smooth muscles, and in proliferating brain cell lines. *J Cell Biol,* 115: 1695-1700 (1991)

30. Deconinck AE, Rafael JA, Skinner JA, Brown SC, Potter AC, Metzinger L, Watt DJ, Dickson G, Tinsley JM and Davies KE. Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. *Cell*, 90: 717-727 (1997)

31. Tinsley JM, Potter AC, Phelps SR, Fisher R, Trickett JI and Davies KE. Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. *Nature*, 384: 349-353 (1996)

32. Deconinck N, Tinsley J, De Backer F, Fisher R, Kahn D, Phelps S, Davies K and Gillis JM. Expression of truncated utrophin leads to major functional improvements in dystrophin-deficient muscles of mice. *Nat Med*, 3: 1216-1221 (1997)

33. Tinsley J, Deconinck N, Fisher R, Kahn D, Phelps S, Gillis JM and Davies K. Expression of full-length utrophin prevents muscular dystrophy in mdx mice. *Nat Med*, 4: 1441-1444 (1998)

34. Gilbert R, Nalbantoglu J, Petrof BJ, Ebihara S, Guibinga GH, Tinsley JM, Kamen A, Massie B, Davies KE and Karpati G. Adenovirus-mediated utrophin gene transfer mitigates the dystrophic phenotype of mdx mouse muscles. *Hum Gene Ther*, 10: 1299-1310 (1999)

35. Yamamoto K, Yuasa K, Miyagoe Y, Hosaka Y, Tsukita K, Yamamoto H, Nabeshima YI and Takeda S. Immune response to adenovirus-delivered antigens upregulates utrophin and results in mitigation of muscle pathology in mdx mice. *Hum Gene Ther*, 11: 669-680 (2000)

36. Chakkalakal JV, Harrison M, Carbonetto S, Chin E, Michel RN and Jasmin BJ. Stimulation of calcineurin signaling attenuates the dystrophic pathology in mdx mice. *Hum Mol Genet*, 13: 379-388 (2004)

37. Suzuki N, Motohashi N, Uezumi A, Yoshimura T, Itoyama Y, Aoki M, Miyagoe-Suzuki Y

and Takeda S. NO production results in suspension-induced muscle atrophy through dislocation of neuronal NOS. *J Clin Invest,* 117: 2468-2476 (2007)

38. Barton ER, Morris L, Kawana M, Bish LT and Toursel T. Systemic administration of L-arginine benefits mdx skeletal muscle function. *Muscle Nerve,* 32: 751-760 (2005)
39. Dennis CL, Tinsley JM, Deconinck AE and Davies KE. Molecular and functional analysis of the utrophin promoter. *Nucleic Acids Res,* 24: 1646-1652 (1996)
40. Burton EA, Tinsley JM, Holzfeind PJ, Rodrigues NR and Davies KE. A second promoter provides an alternative target for therapeutic up-regulation of utrophin in Duchenne muscular dystrophy. *Proc Natl Acad Sci USA,* 96: 14025-14030(1999)
41. Jimenez-Mallebrera C, Davies K, Putt W and Edwards YH. A study of short utrophin isoforms in mice deficient for full-length utrophin. *Mamm Genome,* 14: 47-60 (2003)
42. Takahashi J, Itoh Y, Fujimori K, Imamura M, Wakayama Y, Miyagoe-Suzuki Y and Takeda S. The utrophin promoter A drives high expression of the transgenic LacZ gene in liver, testis, colon, submandibular gland, and small intestine. *J Gene Med,* 7: 237-248 (2005)
43. Hirst RC, McCullagh KJ and Davies KE. Utrophin upregulation in Duchenne muscular dystrophy. *Acta Myol,* 24: 209-216 (2005)
44. Galvagni F and Oliviero S. Utrophin transcription is activated by an intronic enhancer. *J Biol Chem,* 275: 3168-3172 (2000)
45. Kalderon D, Roberts BL, Richardson WD and Smith AE. A short amino acid sequence able to specify nuclear location. *Cell,* 39: 499-509 (1984)
46. Ishii A, Hagiwara Y, Saito Y, Yamamoto K, Yuasa K, Sato Y, Arahata K, Shoji S, Nonaka I, Saito I, Nabeshima Y and Takeda S. Effective adenovirus-mediated gene expression in adult

murine skeletal muscle. *Muscle Nerve,* 22: 592-599 (1999)

47. Imamura M and Ozawa E. Differential expression of dystrophin isoforms and utrophin during dibutyryl-cAMP-induced morphological differentiation of rat brain astrocytes. *Proc Natl Acad Sci USA,* 95: 6139-6144 (1998)
48. Couteaux R, Mira JC and d'Albis A. Regeneration of muscles after cardiotoxin injury. I. Cytological aspects. *Biol Cell,* 62: 171-182 (1988)
49. Rando TA and Blau HM. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J Cell Biol,* 125: 1275-1287 (1994)
50. Uezumi A, Ojima K, Fukada S, Ikemoto M, Masuda S, Miyagoe-Suzuki Y and Takeda S. Functional heterogeneity of side population cells in skeletal muscle. *Biochem Biophys Res Commun,* 341: 864-873 (2006)
51. Coso OA, Montaner S, Fromm C, Lacal JC, Prywes R, Teramoto H and Gutkind JS. Signaling from G protein-coupled receptors to the c-jun promoter involves the MEF2 transcription factor. Evidence for a novel c-jun amino-terminal kinase-independent pathway. *J Biol Chem,* 272: 20691-20697 (1997)
52. Gramolini AO, Belanger G and Jasmin BJ. Distinct regions in the 3' untranslated region are responsible for targeting and stabilizing utrophin transcripts in skeletal muscle cells. *J Cell Biol,* 154: 1173-1183 (2001)
53. Miura P, Thompson J, Chakkalakal JV, Holcik M and Jasmin B,J. The utrophin A 5'-untranslated region confers internal ribosome entry site-mediated translational control during regeneration of skeletal muscle fibers. *J Biol Chem,* 280: 32997-33005 (2005)
54. Bonuccelli G, Sotgia F, Schubert W, Park DS, Frank PG, Woodman SE, Insabato L, Cammer

M, Minetti C and Lisanti MP. Proteasome inhibitor (MG-132) treatment of mdx mice rescues the expression and membrane localization of dystrophin and dystrophin-associated proteins. *Am J Pathol*, 163: 1663-1675 (2003)

55. Lescop C, Herzner H, Siendt H, Bolliger R, Hennebohle M, Weyermann P, Briguet A, Courdier-Fruh I, Erb M, Foster M, Meier T, Magyar JP and von Sprecher A. Novel cell-penetrating alpha-keto-amide calpain inhibitors as potential treatment for muscular dystrophy. *Bioorg Med Chem Lett*, 15: 5176-5181 (2005)
56. Courdier-Fruh I, Barman L, Briguet A and Meier T. Glucocorticoid-mediated regulation of utrophin levels in human muscle fibers. *Neuromuscul Disord*, 12 Suppl 1: S95-S104 (2002)

7. Concluding remarks

Duchenne muscular dystrophy (DMD) is an X-linked progressive disorder caused by a defect in the DMD gene, which encodes dystrophin. Utrophin is a 395-kDa cytoskeletal protein with a high degree identity with dystrophin at the amino acid levels and is an autosomal homologue of dystrophin. Overexpression of utrophin is known to be ameliorated the dystrophinc phenotype muscle in mice. However, the regulatory mechanism of the utrophin gene containing the A-utrophin core promoter has not been elucidated. According to the recent study, the 5.4 kb 5'-flanking region of the utrophin gene containing the A-utrophin core promoter did not drives transgene expression in heart and skeletal muscle. In order to clarify the regulatory mechanism of utrophin expression, therefore, we generated a nuclear localization signal-tagged LacZ transgenic (Tg) mouse, in which the LacZ gene was driven by the 129 bp downstream utrophin enhancer (DUE) and the 5.4 kb 5'-flanking region of the utrophin promoter. The levels of transgene mRNA expression in several tissues were examined by reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (q-RT-PCR), and cryosections of several tissues were stained with hematoxylin and eosin (H&E) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The results obtained in the present study are summarized as follows.

1. *In vivo* study

1) The transgene expression patterns were consistent with endogenous utrophin in several tissues including heart and skeletal muscle.

2) Transgene expression was also up-regulated more in regenerating muscle than in

non-regenerating muscle.

3) Utrophin expression was augmented in the skeletal muscle of DUE Tg/X chromosome-linked muscular dystrophy (*mdx)* mice through cross-breeding experiments.

2. *In vitro* study

1) We established cultures of primary myogenic cells from DUE Tg mouse and found that utrophin up-regulation during muscle differentiation depends on the DUE motif.

2) MEF2C is one of the candidate factor to regulate utrophin expression via DUE because of Endogenous utrophin expression patterns coincided with expression profile of MEF2C.

These results show that DUE is indispensable for utrophin expression in skeletal muscle and heart, and primary myogenic cells from this Tg mice provide a high through-put screening system for drugs that up-regulate utrophin expression.

8. Publication list

1）Shirato K, Motohashi N, Tanihata J, Tachiyashiki K, Tomoda A and Imaizumi K (2006) Effects of two types of inactivity on the number of white blood cells in rats. *European Journal of Applied Physiology*, 98:590-600

2）Shirato K, Tanihata J, Motohashi N, Tachiyashiki K, Tomoda A and Imaizumi K (2007) Beta2-agonist clenbuterol induced changes in the distribution of white blood cells in rats. *Journal of Pharmacological Sciences*, 104 (2):146-52

3）Tanihata J, Suzuki N, Miyagoe-Suzuki Y, Imaizumi K and Takeda S (2008) Downstream utrophin enhancer is required for expression of utrophin in skeletal muscle. *Journal of Gene Medicine*, 10 (6): 702-713

9. Oral presentation list

1）Oyama K, Wada T, Tachiyashiki K, Shirato K, Tanihata J, Motohashi N, Higashino Y, Suzuki K and Imaizumi K: Comparisons among three life-stages in capsaicin-induced changes of plasma energy substrate levels in rats. *Japanese Journal of Physiology,* 54 (Supplement): S232 (2004)

2）Motohashi N, Tanihata J, Tachiyashiki K and Imaizumi K: *In vivo* effect of hydrocortisone on plasma energy substrate levels in rats. *Japanese Journal of Physiology,* 54 (Supplement): S233 (2004)

3）Tanihata J, Motohashi N, Tachiyashiki K and Imaizumi K: *In vivo* effect of synthesized glucocorticoid, dexamethasone on plasma energy substrate levels in rats. *Japanese Journal of Physiology*, 54 (Supplement): S233 (2004)

4）Adachi S, Tachiyashiki K, Tanihata J, Motohashi N and Imaizumi K: Effect of agonist, clenbuterol on plasma substrate, insulin and cyclic AMP levels in rats. *Japanese Journal of Physiology*, 54 (Supplement): S234 (2004)

5）Shirato K, Tachiyashiki K, Motohashi N, Tanihata J, Wada T, Ichinose T and Imaizumi K: Effects of hypokinesia/hypodynamia and agonist on white blood cell levels in rats. *Japanese Journal of Physiology,* 55 (Supplement): S110 (2005)

6）Tanihata J, Tachiyashiki K, Motohashi N, Shirato K, Wada T, Ichinose T and Imaizumi K: Effects of skeletal muscle hypertrophy and atrophy on muscle DNA, RNA and protein levels. *Japanese Journal of Physiology,* 55 (Supplement): S225 (2005)

7）Motohashi N, Tachiyashiki K, Tanihata J, Ichinose T and Imaizumi K: Muscle atrophy-induced changes of cathepsin and dipeptide levels in rats. *Japanese Journal of Physiology*, 55 (Supplement): S225 (2005)

8）Shirato K, Tanihata J, Motohashi N, Tachiyashiki K, Tomoda A and Imaizumi K: Effect of β_2-agonist, clenbuterol on the number of white blood cell in rats. *FASEB Journal*, 21 (6): A936 (2007)

9）Shirato K, Motohashi N, Tanihata J, Tachiyashiki K, Tomoda A and Imaizumi K: Two types of inactivity-induced changes of the number of white blood cells in rats. *FASEB Journal*, 21 (6): A1392 (2007)